Title: Platelets kill circulating parasites of all major *Plasmodium* species in human malaria

Short title: Platelets kill malaria parasites in humans

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Key points:

- Platelets directly interact with and kill circulating *Plasmodium* parasites in malaria patients to help control parasitemia.
- *In* vitro platelet anti-plasmodicidal activity against *P. knowlesi* involves platelet-cell binding and intracellular accumulation of PF4.

Abstract: Platelets are understood to assist host innate immune responses against infection, although direct evidence of this function in any human disease, including malaria, is unknown. Here we characterized platelet-erythrocyte interactions by microscopy and flow cytometry in malaria patients naturally infected with *Plasmodium falciparum*, *P. vivax*, *P. malariae* or *P. knowlesi*. Blood samples from 376 participants were collected from malaria-endemic areas of Papua, Indonesia, and Sabah, Malaysia. Platelets were observed binding directly with and killing intraerythrocytic parasites of each of the *Plasmodium* species studied, particularly mature stages, and was greatest in *P. vivax* patients. Platelets preferentially bound to infected more than uninfected erythrocytes in the bloodstream. Analysis of intraerythrocytic parasites indicated the frequent occurrence of platelet-associated parasite killing, characterized by the intraerythrocytic accumulation of platelet factor-4 and terminal deoxynucleotidyl transferase dUTP nick-end labeling of parasite nuclei ($PF4^+TUNEL^+$ parasites). These $PF4^+TUNEL^+$ parasites were not associated with measures of systemic platelet activation. Importantly, patient platelet counts, infected erythrocyte-platelet complexes and platelet-associated parasite killing correlated inversely with patient parasite loads. These relationships, taken together with the frequency of platelet-associated parasite killing observed amongst the different patients and *Plasmodium* species, suggest that platelets may control the growth of between 5 and 60% of circulating parasites. Platelet-erythrocyte complexes comprised a major proportion of the total platelet pool in malaria patients and may therefore contribute considerably to malarial thrombocytopenia. Parasite-killing was demonstrated to be platelet factor-4-mediated in *P. knowlesi* culture. Collectively, our results indicate that platelets directly contribute to innate control of *Plasmodium* infection in human malaria.

Keywords: platelets, malaria, *Plasmodium*, killing, falciparum, vivax, malariae, knowlesi, platelet factor-4, thrombocytopenia.

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Introduction

Platelets are the second most abundant cell of the circulation after red blood cells (RBC) and the principle regulators of hemostasis. Platelets can also integrate host immune responses through production of immunomodulatory molecules and via cell-to-cell interactions with white blood cells (WBC), and may have host-protective roles in infectious disease.[1] Platelets are an abundant source of antimicrobial molecules[2,3], have broad-spectrum pathogen killing activities[4-9] and are required for host-mediated pathogen control and host survival in some infectious disease models.[10-13] Clinically, low platelet counts are often associated with a poor prognosis and increased risk of infection.[14-16] However, to date direct evidence that platelets contribute to host protection in any human infectious disease is lacking.

Malaria kills over 400 000 people each year.[17] While most deaths are caused by *Plasmodium falciparum*, all *Plasmodium* species can cause severe and fatal infection.[18-21] Malaria pathogenesis is driven primarily by parasite biomass[22-24] and modulated by host innate and adaptive immune responses.[25,26] Thrombocytopenia is common in all malarias and is a risk factor for mortality in African children with falciparum malaria[27], Southeast Asian adults and children with falciparum and vivax malaria[28], and adults with knowlesi malaria.[21] However, the mechanisms leading to thrombocytopenia are not fully understood and its subsequent effects on parasite biomass, disease control or progression has yet to be quantified for any of the human *Plasmodium* species.

Indirect evidence from separate sources suggests that platelets may contribute to the host protection in malaria. This includes observations of human platelets directly binding to and killing *P. falciparum*-infected RBC (iRBC) in culture, and reduced survival in *Plasmodium*-infected mice depleted of platelets.[12,13,29-31] A mechanism for the direct killing of *Plasmodium*

involves platelet factor-4 (PF4), an abundant antimicrobial protein secreted by platelets, that, upon entering the cell via the Duffy-antigen – a chemokine receptor expressed by RBC[30] – kills *P. falciparum* parasites by selectively lyzing the parasite digestive vacuole.[29] However, a recent study did not reproduce the parasite killing effect of platelets in *P. chabaudi*-infected mice or *P. falciparum*-iRBC[32], showing the difficulties in using *in vitro* and *in vivo* disease models to study these phenomena[33], and highlighting the need for additional research in people with malaria.[34]

No clinical studies have addressed the role of platelets in killing *P. falciparum* parasites across the spectrum of clinical disease, nor their role in protection and pathogenesis in human malaria from non-falciparum *Plasmodium* species. Here we characterized cell-to-cell platelet interactions in patients naturally infected with *P. falciparum*, *P. vivax*, *P. malariae* or *P. knowlesi*, and examined relationships between platelet-associated parasite killing and parasite biomass. We also demonstrate the mechanism by which human platelets can kill *P. knowlesi*, a second culturable human *Plasmodium* species.

Methods

Study participants

In Papua, malaria patients attending the Mitra Masyarakat Hospital in Timika were enrolled between 2014-2016. This lowland forest region has perennial transmission of *P. falciparum*, *P. vivax* and *P. malariae*. In Sabah, patients were enrolled between 2012-2016 from 3 district hospitals in Kudat Division as part of concurrent prospective clinical studies.[35] Sabah is an area of low malaria transmission, which during this period was co-endemic for *P. falciparum, P. vivax,* and the zoonotic parasite, *P. knowlesi*.

Criteria for enrolment in both cohorts included: a blood film positive by microscopy for any *Plasmodium* species, including mixed infections in Papua, fever or history of fever in the last 48 h, no major concurrent illness or co-morbidity, and no prior antimalarial therapy in the preceding 24 h. Patients were excluded if pregnant or lactating. In Papua patients aged <16 or >60 years or with a hemoglobin concentration ≤7 g/dL were also excluded. Severe malaria was defined according to WHO 2014 research criteria.[20] Hospitalization was at the discretion of the treating clinician in Papua and was mandatory in all malaria patients in Sabah. All malaria patients were treated according to local guidelines as described previously.[20,36-38] Controls were selected from visitors or relatives of malaria patients, with no fever or history of fever in the preceding 14 d and with a blood film negative for malaria parasites.

Details of blood collection and analysis procedures are described in Supplementary Materials.

Platelet binding to iRBC and uRBC

Blood samples were analyzed by flow cytometry to measure platelet-bound iRBC or uninfected RBC (uRBC), and expressed as frequencies and absolute numbers. In Papua, assays were performed on fresh samples and in Sabah on fixed blood (Cytofix™, BD Biosciences). Fluorescent antibody panels comprised anti-CD45 (white cell exclusion marker), anti-CD41 or CD42b (platelet marker), anti-CD235ab (RBC marker), and either Hoechst 33342 or DRAQ-5 (parasite nuclear marker). The gating strategy for measuring platelet-iRBC/uRBC complexes in Papua is illustrated in **Fig. 1B** and in Sabah in **Fig. S1A**. Refer to Supplementary Materials for details of the staining materials and procedures.

Platelet-associated parasite killing

Immunofluorescent microscopy of Cytofix™-treated blood was used to quantify platelet-associated parasite killing, based on a terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) assay described elsewhere[30] that labels degraded or sheared DNA, an indication of apoptosis or necrosis. Platelets and intraerythrocytic localization of PF4 were detected with an anti-human PF4 Ab (Abcam, UK) and intraerythrocytic *Plasmodium* DNA was identified using DAPI. Only high parasitemia samples (>5000 parasites/μL) were analyzed and the number of parasites counted per sample recorded. In a subset of patients, iRBC were distinguished into rings ($DAPI^+$ without parasite pigment) or mature stages ($DAPI^+$ with parasite pigment) by immunofluorescence, and the proportion of stages confirmed with Giemsa-stained blood smears performed in parallel. Refer to Supplementary Materials for more detail.

Platelet activation

In Papua, platelet activation markers including platelet PAC-1 binding (recognizing the glycoprotein IIb/IIIa complex of activated platelets), P-selectin (CD62P) surface expression, and platelet-monocyte and platelet-neutrophil aggregate formation, were measured using two flow cytometry panels. Plasma PF4 concentrations were measured by ELISA. In Sabah, platelet-WBC aggregates were determined from the RBC-binding panel. The platelet activation gating strategies are illustrated in **Fig. S1, B** and **C**. Refer to Supplementary Materials for details of the staining and analysis procedures.

In vitro parasite and platelet experiments

P. knowlesi and *P. falciparum* were cultured in 2.5% hematocrit O+ human RBC as described elsewhere.[39,40] Platelets were purified from freshly collected human blood as described previously.[12] Refer to Supplementary Materials for details of the culture conditions and platelet preparation.

Platelet count and quiescence were determined using an ADVIA 2120i hematology analyzer (Siemens, Australia). Platelet activation capacity was tested by treating platelets with 1 U/mL of human thrombin for 10 min, then measuring PAC-1 binding and P-selectin surface expression by flow cytometry. All platelet preparations in this study (8 preparations from six donors) satisfied the criteria for quiescence (mean platelet volume <9 fL and mean platelet component >23 g/dL) and activation capacity (>1000-fold and >250-fold increase in PAC-1 and CD62P MFI, respectively, compared to untreated rested platelets) (**Fig. S4**).

For the parasite-platelet co-culture experiments, *P. falciparum* parasites were synchronized for ring stages the day before experiments using 5% (w/v) D-sorbitol.[41] Late-stage *P. knowlesi* and *P. falciparum* trophozoites were purified on the day of experiment using 70% Percoll® gradient

centrifugation (3500 RPM without brake for 10 min) and washed twice with complete culture medium (CCM). Parasitemia was adjusted to 0.5% at 2% hematocrit and platelets added; equal volumes of Tyrode's buffer were added to untreated controls. In some experiments before adding to the parasites, recombinant human PF4 (Peprotech, US) and platelet lysates were incubated with 0.5 mg/mL of preservative-free rabbit anti-human PF4 Ab (Abcam, UK) or preservative-free rabbit serum (Novus Biologicals, US) for 15 min at 4°C. For transwell experiments, parasites were separated from 50 million platelets/mL, platelet lysate or 0.5 μM PF4 using transwell inserts with 0.2 μm pore size (Anopore™ membrane, Nalge Nunc International, Denmark). The well inserts were pre-soaked with CCM prior to use. *P. knowlesi* was harvested after 24 h, and *P. falciparum* after 48 h culture with platelets, lysate or protein. Giemsa-stained thin smears were prepared, and 1000 RBC were counted per slide at 1000x magnification. Percent parasite growth was calculated using the formula below.

% Parasite Growth = [(treatment parasitemia – initial parasitemia) ÷ (untreated parasitemia – initial parasitemia)] x 100

Platelet binding to parasites was determined in 24 h cultures containing 2% asynchronous parasites and 60 million platelets/mL. Harvested co-cultures were fixed in diluted Cytofix™ (Biosciences, Australia) for at least 24 h and stored at 4°C. For flow cytometry quantitation, fixed cells were washed once with 1% (w/v) BSA/PBS, then stained with mouse anti-human CD42b conjugated to phycoerythrin and mouse anti-human CD235ab conjugated to allophycocyanin for 20 min at 4°C, followed by 5 μg/mL Hoechst 33342 for 5 min at 4°C. Fluorescence signals were measured using the LSR Fortessa (BD Biosciences). At least 100 000 events were collected per sample.

Statistics

The Mann-Whitney test or the Kruskal-Wallis test followed by Dunn's multiple comparisons was used for between-group comparisons of clinical data. The Wilcoxon matched-pairs signed rank test was applied to paired datasets. One-way ANOVA with Sidak's multiple comparisons was used for *in vitro* data. Associations between two variables were assessed using Spearman correlation. Bonferroni corrections to *P*-values were applied where appropriate. Data were log transformed for multivariate comparisons. All statistical analyses were performed using GraphPad Prism 7 (GraphPad Software, La Jolla, CA) or Stata 14 (StataCorp, Texas, US).

Study approval

The study was approved by the Human Research Ethics Committees of Gadjah Mada University, Indonesia (Papua), the Malaysian Ministry of Health (Sabah), Menzies School of Health Research, Australia (Papua and Sabah), the Macquarie University and the Australian National University. Written informed consent was obtained from all participants.

Results

Study participants

Separate cohorts of malaria patients and healthy controls were enrolled in Papua, Indonesia (n=143), and Sabah, Malaysia (n=233). Patients were categorized according to *Plasmodium* species, and baseline characteristics, including parasitemia, platelet counts and disease severity presented (**Tables 1 and 2**). The majority of malaria patients at each site had non-severe malaria, 92% in Papua and 97% in Sabah. Relative to controls, platelet counts were significantly lower in malaria from all *Plasmodium* species at both study sites and correlated inversely with parasitemia in the *P. vivax* groups (Papua, r=-0.37, p=0.022, n=38; Sabah, r=-0.36, p=0.0008, n=84) and with histidine-rich protein-2 (HRP2) in falciparum malaria in Papua (r=-0.45, *p*=0.0007, n=54).

Platelet-iRBC and platelet-uRBC complexes are formed in the circulation of malaria patients

Examination of patient thin blood smears revealed platelet binding to both iRBC and uRBC. Platelets were bound to all asexual parasite stages in each of the *Plasmodium* species, with evidence of platelet aggregates surrounding *P. vivax*-iRBC (**Fig. 1A**). Characteristic features of dying parasites within platelet-bound iRBC were observed, including spread of parasite pigment suggesting dissolution of the digestive vacuole.

Flow cytometry was used to quantify the proportions of uRBC and iRBC that were each bound to platelets, hereafter platelet-uRBC and platelet-iRBC complexes (**Figs. 1B** and **S1A**). Proportions of platelet-uRBC were similar amongst all the patient groups (median range: 0.10%

– 0.19%) and were significantly lower than the controls (0.28% and 0.42%; **Fig. 1, Ci** and **Cii**, respectively). In contrast, greater proportions of platelet-iRBC were observed in all the patient groups (**Fig. 1, Di** and **Dii,** and **Table S1**). The greatest proportions were observed in *P. knowlesi* patients (median 18.1%), which was significantly higher than in Sabah *P. falciparum* patients (median 6.3%; p=0.002, **Table S1**); Overall complex magnitudes were lower in the Papua than the Sabah groups, which may be attributable to differences in sample preparation and staining. However, for all groups platelet-iRBC complexes were significantly greater than platelet-uRBC complexes. Compared as a ratio, the proportions of platelet-iRBC complexes were between eight and almost 100-fold greater than platelet-uRBC complexes (**Table S1**). We also separately analyzed the subgroup of 28 Sabah patients with *P. vivax* or *P. knowlesi* malaria who were children aged 12 or younger (**Table 2**); similar proportions of platelet-iRBC/uRBC complexes to adults, and significantly greater frequencies of platelet-iRBC versus platelet-uRBC were observed in these groups (**Fig. S3, A–C**).

Using the proportions of the complexes and the respective RBC count in each individual, we calculated concentrations of platelet-RBC complexes (iRBC and uRBC combined). The medians ranged between 3,100 and 13,600/μL blood (**Table 3**). Compared to respective circulating levels of free platelets (i.e. free platelet:platelet-RBC complex ratio; **Table 3**), the complexes comprise a substantial proportion of the total platelet pool. For example, in *P. knowlesi*-infected patients, on average, one complex was predicted to exist for every 7 non-complexed platelets. The highest ratio, in *P. malariae*, predicted one complex for every 19 platelets (**Table 3**). By comparison, free platelet:platelet-WBC ratios were much higher (≥118), indicating these complexes were only a minor component of the platelet pool (**Table 3**).

Correlative analyses revealed inverse relationships between platelet-iRBC complexes and parasitemia; these were significant in malaria from each species, except *P. malariae* (**Fig. 1, Ei, Eii** and **Fig. S2A**). After adjusting for platelet count, these relationships remained significant for *P. vivax* and *P. knowlesi* (both $p<0.0001$). No significant relationships were observed between parasitemia and platelet-uRBC complexes. There was no consistent relationship between complexes and hemoglobin levels.

Taken together, these data show that platelets bind and form stable complexes with RBC, with a greater preference for iRBC than uRBC. The frequencies of these complexes in the circulation of patients and controls are substantial and compared to free platelets comprise a relatively large proportion of the total platelet pool. In addition, the proportions of platelet-iRBC complexes are higher in patients with low levels of parasites, and lower in patients with high parasite burden.

Platelets directly kill parasites in the circulation of malaria patients

We conducted TUNEL and PF4 immunostaining on patient blood to detect and quantify the frequencies of dead intraerythrocytic parasites and the co-occurrence of PF4 accumulation within the iRBC. With these methods, iRBC containing PF4 and TUNEL labeling ($PF4^+TUNEL^+$) are indicative of direct platelet contact with the cell (by uptake of platelet-produced PF4) and consequent PF4-mediated killing of the parasite.[29,30] We observed $PF4^+TUNEL^+$ iRBC in all *Plasmodium* species in both patient cohorts (**Fig. 2A**). Quantification of these observations showed substantial variances in the proportions of $PF4^+TUNEL^+$ iRBC, although the medians calculated for each species were similar, except for *P. vivax*, which was significantly greater in both Papua and Sabah compared to the other species (**Fig. 2B** and **Table 4**). Notably, PF4 was present in more than half of the $TUNEL^+$ (dead) parasites; in Papua, a median of 96% of

TUNEL$^+$ *P. vivax*-iRBC contained PF4, which was significantly greater than the 52% median observed in *P. falciparum* (p<0.0001) and 69% median in *P. malariae* (p=0.051) (**Fig. 2C**). No PF4 staining or TUNEL labeling was observed in enriched suspensions of uninfected reticulocytes. The proportion of PF4$^+$TUNEL$^+$ iRBC correlated inversely with parasitemia in Papuan patients infected with *P. vivax* (r=-0.42, p=0.020) and *P. falciparum* (r=-0.36, p=0.010); no significant relationships were observed in the other cohort groups, in which fewer samples were examined (**Fig. 2D** and **Fig S2B**).

We compared PF4$^+$TUNEL$^+$ frequencies in young (ring) versus mature (trophozoite and schizont) asexual stage parasites (**Fig. 2E** and **Table 4**). In the Papuan *P. vivax* samples, PF4$^+$TUNEL$^+$ mature-stage parasites outnumbered ring-stages almost ten times (63.6% vs 6.4%; p<0.0001); there was a similar significant difference in *P. malariae* (6.3% vs 0%; p=0.007), but not in *P. knowlesi* (14.9% vs 10.8%; p=0.354). Only ring-stage parasites were observed in the *P. falciparum* samples, consistent with the ability of mature stages to sequester in tissues. There were no significant differences in the proportions of PF4$^+$TUNEL$^+$ rings between patient location or parasite species (**Fig. 2F**). All ring-stage parasites (i.e. TUNEL$^+$ and TUNEL$^-$) were also categorized according to PF4 staining; the greatest proportions were observed in Papuan *P. vivax* samples (median 43.8%), which was significantly greater than in *P. falciparum* or *P. malariae* (median 12.0% and 9.1%, respectively; **Fig. 2G**).

Collectively these data show that, irrespective of species, a substantial proportion of *Plasmodium* accumulate platelet-derived PF4 and undergo intraerythrocytic death, most likely as a consequence of the known cytotoxic actions of PF4.

Platelet-derived PF4 accumulation in iRBC and parasite killing are not associated with systemic platelet activation

We observed that not all PF4-stained parasites were bound by platelets (Range 0-25%) and some platelet-bound cells were not stained for PF4 (Range 0-5.9%; **Table S2**). We determined if systemic platelet activation contributes to absorption of plasma PF4 into the iRBC. None of our markers for platelet activation, including plasma PF4 concentrations, platelet surface expression of CD62P (P-selectin) and PAC-1 (activated GPIIb/IIIa) on circulating platelets, were significantly elevated in any of the patient groups (**Table S3**). In addition, no relationships were observed that supported a role for systemic platelet activation in the iRBC accumulation of PF4 (**Table S4**).

Cultured *P. knowlesi* and *P. falciparum* are sensitive to human platelets and PF4

In vitro platelet killing of *P. falciparum* has been demonstrated previously[12,29-31] but not for other human *Plasmodium* species. We therefore co-cultured *P. knowlesi* with or without different concentrations of washed and rested human platelets and measured effects on parasite growth over 24 h. We observed a significant and platelet concentration-dependent reduction in parasite growth, maximal at 75 million platelets/mL (~60% reduction) (**Fig. 3A**). Implementation of platelet-to-iRBC ratios and parasitemias comparable to clinical settings, and strict control of platelet quality (**Fig. S4**) were critical components of our experimental system. A similar significant growth inhibition effect was also observed in *P. falciparum* cultures treated with the same platelet preparations (**Fig. 3B**). *P. knowlesi* was sensitive to recombinant human PF4 (IC_{50} ~ 0.5 μM), and to treatment with platelet lysates, which was blocked by inclusion of anti-PF4 antibodies (**Fig. 3, C** and **D**). Platelet inhibition of parasite growth was prevented when platelets

and *P. knowlesi*-iRBC were physically separated in co-cultures using cell-impermeable transwells, but remained sensitive to platelet lysate and PF4 (**Fig. 3E**), indicating that direct platelet-cell contact and platelet-derived PF4 are responsible for the cytotoxic effects.

Platelets were observed physically bound to *P. knowlesi*-iRBC (**Fig. 3F**). The frequency of platelet-iRBC complexes was twice of platelet-uRBC complexes in both *P. knowlesi* and *P. falciparum* after 24 h incubation with platelets and was significant for *P. knowlesi* ($p<0.05$; **Fig. 3G**). Platelet-treated *P. knowlesi* cultures contained significantly greater proportions of $TUNEL^+$ iRBC; the majority of these iRBC were also $PF4^+$ (**Fig. 3, H** and **I**). PF4 was not detected in untreated iRBC (**Fig. 3H**). Collectively, human platelets can kill both *P. falciparum* and *P. knowlesi* asexual blood-stage parasites under well-defined culture conditions. The killing mechanism requires platelet-cell contact and PF4; intraerythrocytic PF4 is cytotoxic to *P. knowlesi*.

Discussion

Here we demonstrate for the first time in humans that platelets can directly kill a microbial pathogen, erythrocytic stage *Plasmodium*. Platelets were observed bound to *Plasmodium*-iRBC of all four of the major species that cause human disease, *P. falciparum*, *P. vivax*, *P. malariae* and *P. knowlesi*. Platelet binding to uRBC was also observed, although the proportions of these were significantly lower than platelet-iRBC complexes. In addition, the proportions of platelet-iRBC complexes were inversely related to parasite burden, suggesting a potential cause and effect relationship between cell-cell binding and parasite growth. We observed frequent intracellular accumulation of PF4 in these iRBC and death of the intraerythrocytic parasites. Further evidence of platelet's cytotoxic mechanism of action against parasites was obtained using cultures of *P. knowlesi* and *P. falciparum*. The frequent occurrence of platelet-associated killing of parasites in the periphery of malaria patients, and its inverse correlation with parasite load, significant at least for *P. falciparum* and *P. vivax*, suggests the importance of platelets in the host control of parasites. Collectively, our data predict that platelets may kill as many as 5-20% of circulating blood-stage *Plasmodium* in clinical malaria, and in *P. vivax* this may be as high as 60%.

We have previously proposed a mechanism of PF4 accumulation and parasite killing involving direct platelet-iRBC contact followed by local release of PF4 and uptake into the parasite via the Duffy-antigen.[30] Here, our *in vitro* studies using transwell filters to separate platelets and *P. knowlesi* parasites demonstrated that direct platelet-cell contact is necessary for parasite killing, which confirms previous studies.[31] Our cumulative *in vitro* data contrast with recent findings reported by Gramaglia and colleagues[32], who found no parasite growth inhibition by platelets in *P. falciparum* cultures. We predict this is due to differences in experimental design, noting

especially that *in vitro* parasite killing by platelets is only observed in cultures containing physiologically relevant proportions of parasite-infected cells (<1%, in this study and previously [12,31]). The aforementioned study also did not observe changes in parasite growth rates when platelet levels were altered in *Plasmodium*-infected mice, or evidence that platelets were required for survival, which conflicts the findings of others.[12,13] This could be due to differences in the *Plasmodium* strains used amongst the studies, which can affect parasite virulence, growth and sequestration, and host response characteristics. In addition, recognizing the definitive parasite killing activity of platelets in the circulation requires distinguishing dead parasites from healthy parasites, which are greatest in platelet sufficient mice[12] and in parasitized cells that contain platelet-derived PF4 (this study and [30]). In the patient studies we also considered, and excluded, the possibility that PF4 is absorbed by circulating parasites from the plasma. Systemic platelet activation was negligible and plasma PF4 concentrations were approximately 1000-fold lower than *in vitro* concentrations required for *Plasmodium* killing. We also observed substantially higher frequencies of PF4 accumulation in mature-stage *P. vivax*-iRBC and greater rates of parasite death. Accumulation may be determined by the higher Duffy-antigen levels expressed on reticulocytes[42-44], for which *P. vivax* has an exclusive tropism.[45] While circulating *P. vivax* parasitemia is intrinsically limited by the number of circulating reticulocytes, we speculate that the greater degree of platelet-associated killing observed in *P. vivax* infection may be a substantial additional contributor to the lower parasitemia generally observed in these patients compared to *P. falciparum*.[18,46,47]

Platelet cytoadherence to the endothelium and WBC are well-established pathological drivers of vascular and inflammatory diseases. However, the occurrence and consequences of platelets interacting with RBC in any disease setting have been under-reported.[48-50] Our unique ability to

systematically characterize platelet-RBC interactions revealed that platelets have a greater capacity to bind *Plasmodium*-iRBC versus uRBC. This may be partly determined by parasite-expressed proteins present on the RBC surface, such as the *P. falciparum* cell adhesion molecule PfEMP1 shown previously to mediate platelet binding through CD36.[51] Platelet binding to uRBC may be mediated through erythroid-expressed ICAM-4 and platelet GPIIb/IIIa.[52] The identities and roles of other RBC and platelet molecules involved in platelet-RBC binding, especially in the other *Plasmodium* species, remain to be determined.

Platelet-RBC complexes may have a role in malaria-induced thrombocytopenia, which has been reported in many other clinical studies[53-55], and was also evident in our patient groups. The underlying causes of thrombocytopenia have been variously attributed to systemic platelet activation, immune-mediated clearance and vascular pooling.[56-61] Platelets complexed with RBC are not recognized by hematological analyzers, thus complex formation would lead to an apparent platelet loss. The high frequencies of these complexes relative to free platelets in our patients suggest they comprise a substantial proportion of the total platelet pool. If there is an accelerated turnover of these complexes, this would further enhance the contribution of complexes to platelet loss. Interestingly, host mechanisms that remove diseased and damaged circulating cells such as the spleen are upregulated in malaria[62]; the lifetime of platelet-RBC complexes in the circulation remains unknown. Further supporting this hypothesis, platelet-RBC complexes were greatest in our knowlesi malaria patients and this species causes the highest frequency of thrombocytopenia.[35,53]

Platelet activation and binding is considered a key mechanism in enabling sequestration of *P. falciparum*-iRBC to microvasculature and platelet-mediated iRBC sequestration is associated with fatal outcome in cerebral malaria studies.[63-66] Our data in predominantly non-severe *P.*

falciparum, *P. vivax*, and *P. malariae* indicated negligible systemic platelet activation. Systemic platelet activation in severe and non-severe falciparum malaria were comparable, however, numbers were small and we cannot exclude greater systemic platelet activation in human severe malaria[67,68], nor can we exclude tissue platelet activation not detected by the circulating measures used in this study.

Overall, our study demonstrates the direct pathogen-killing actions and host protective roles of platelets during human malarial infection. Given platelets show broad-spectrum anti-microbial activity, and the risks of infection associated with thrombocytopenia and platelet disorders are elevated, a general role for platelets in the innate host defence against microbial infection should be considered.

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Author contributions

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Competing interest

The authors declare that no competing interest exists.

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Supplementary materials list

Supplementary methods

Blood collection and parasite biomass

Platelet binding to iRBC and uRBC

Platelet-associated parasite killing

Platelet activation

Parasite culture

Platelet purification

Culture medium and buffers

Fig. S1. Representative gating strategies

Fig. S2. Platelet binding and killing correlations with parasitemia

Fig. S3. Platelet binding in Sabah children

Fig. S4. Analysis of purified human platelet preparations

Table S1. Platelet-RBC complexes

Table S2. Platelet binding to PF4-stained and PF4-unstained infected RBC

Table S3. Platelet activation markers in malaria patients and healthy controls

Table S4. Association between platelet activation and platelet-RBC complexes, $TUNEL^{+}PF4^{+}$ iRBC and indicators of disease severity in the Papua cohort

Tables

Table 1. Baseline characteristics of n=143 malaria patients and healthy controls from Papua

	Controls n =24	***P. falciparum*** n=55	***P. vivax*** n=38	***P. malariae*** n=14	**Mixed infection** n=12 [a]	Kruskal-Wallis test *p*-value
Age, yr	28 [26-33.8]	29 [19-37]	24 [19-32.8]	33.5 [26-47]	22 [19.3-33]	0.027
Males, *n* (%)	10 (42%)	29 (53%)	23 (61%)	4 (29%)	7 (58%)	-
Ethnicity, Highland/ Lowland/non-Papuan, *n*	12/11/1	50/2/3	33/3/2	14/0/0	12/0/0	-
n with severe malaria [b]	-	8	1	-	-	-
Parasitemia (parasites/μL)	Not detected by microscopy	15,600 [3,660-105,000] [c]	6,160 [2,120-11,400]	1,230 [815-2,540]	7,160 [4,590-106,000]	<0.0001
Hemoglobin (g/dL)	12.3 [11.5-14.6]	12.4 [10.2-13.4]	11.9 [10.5-13.1]	9.6** [8.6-10.6]	11.7 [8.8-13.3]	0.0007
WBC count (x1000/μL)	7.4 [6.2-8.2]	5.1** [3.7-6.6]	5.5 [4.8-7.5]	4.2** [3.2-5.1]	5.8 [4.4-7.2]	<0.0001
Monocyte count (x1000/μL)	0.45 [0.42-0.55]	0.53 [0.30-0.77]	0.54 [0.40-0.67]	0.54 [0.37-0.68]	0.51 [0.20-0.66]	0.780
Neutrophil count (x1000/μL)	3.5 [2.5-4.4]	2.8 [2.0-4.8]	3.8 [2.8-4.3]	1.6* [1.5-2.4]	3.9 [2.6-4.8]	0.0006
Platelet count (x1000/μL)	202 [171-228]	66** [39-99] [d]	78** [53-113]	80** [56-101]	60** [44-120]	<0.0001

Footnotes:

All values are median [interquartile range] unless otherwise indicated.

Kruskal-Wallis with Dunn's multiple comparisons test, significantly different to controls (**p<0.0005, *p<0.005).

[a] Mixed infection of *P. falciparum* and *P. vivax*.

[b] Severe malaria criteria encountered: cerebral malaria (Glasgow Coma Score ≤10 for >30 min), jaundice (visible jaundice OR creatinine >1.5 mg/dL OR bilirubin >3 mg/dL AND >100,000 parasites/μL), acute renal failure (creatinine >3 mg/dL +/- urine output <400 mL/day OR urea > 20 mM), hypoglycemia (plasma glucose <40 mg/dL), hyperparasitemia (asexual parasitemia >10%), hypotension (blood pressure <80 mmHg AND cool peripheries), and respiratory distress (respiratory rate >30/min AND O_2 saturation <92%).

[c] Median parasitemia in severe (283,000 parasites/μL) versus non-severe *P. falciparum* (10,800 parasites/μL; Mann-Whitney test p=0.0009).

[d] Median platelet count in severe (18,500/μL) versus non-severe *P. falciparum* (74,000/μL; Mann-Whitney test p<0.0001).

Table 2. Baseline characteristics of n=233 malaria patients and healthy controls from Sabah

	Controls n =28	***P. falciparum*** n=14	***P. vivax*** n=85	***P. knowlesi*** n=106	Kruskal-Wallis test *p*-value
Age, yr	31.5 [26-36.8]	25 [18-37.8]	22 [12-34]**	34 [22-52.3]	<0.0001
n aged ≤12 yr	-	-	22	6	-
Males, *n* (%)	10 (36%)	12 (86%)	62 (73%)	84 (79%)	-
n with severe malaria [a]	-	1	0	6	-
Parasitemia (parasites/μL)	Not detected by PCR	9,850 [3,130-21,800]	4,640 [1,830-7,630]	2,650 [648-8,770] [b]	0.025
Hemoglobin (g/dL)	13.7 [12.9-14.8]	12.9 [11.1-14.8]	12.4** [10.5-13.9]	13.4 [11.9-14.6]	0.002
RBC count (x10^6/ μL)	5.1 [4.9-5.5]	4.9 [4.2-5.3]	4.6 [4.1-5.0]***	5.1 [4.6-5.5]	<0.0001
WBC count (x1000/μL)	7.8 [6.6-8.9]	5.7** [4.1-6.9]	6.4* [5.3-7.9]	6.2** [5.1-7.9]	0.002
Platelet count (x1000/μL)	348 [267-363]	86*** [56-156]	97*** [67-133]	72*** [52-108] [c]	<0.0001

Footnotes:

All values are median [interquartile range] unless otherwise indicated.

Kruskal-Wallis with Dunn's multiple comparisons test, significantly different to controls (***p<0.0005, **p<0.005, * p<0.05).

[a] Severe malaria criteria encountered: acute kidney injury (creatinine >265 μmol/L), significant abnormal bleeding, severe anemia (hemoglobin <7 g/dL or hematocrit <20%), jaundice (visible jaundice OR creatinine >1.5 mg/dL OR bilirubin >3 mg/dL AND

>100,000 parasites/μL [*P. falciparum*] or >20,000/μL [*P. knowlesi*]), hyperparasitemia (asexual parasitemia >10% [*P. falciparum*] OR >100,000/μL [*P. knowlesi*]), metabolic acidosis (HCO_3 <15 mmol/L OR lactate >5 mmol/L).

[b] Median parasitemia in severe (136,000 parasites/μL) versus non-severe *P. knowlesi* (2,450 parasites/μL; Mann-Whitney test $p<0.0001$).

[c] Median platelet count in severe (52,500/μL) versus non-severe *P. knowlesi* (72,000/μL; Mann-Whitney test $p=0.154$).

Table 3. Circulating platelet-RBC and platelet-WBC concentrations and ratios to free platelets

Patient Cohort	*Plasmodium* species	*n* of samples analyzed	Platelet-RBC complexes		Platelet-WBC complexes	
			x10^3 per μL blood [a]	**free platelet : complex ratio** [b]	**x10^3 per μL blood** [c]	**free platelet : complex ratio** [d]
Papua	Controls	17	13.6 [10.1-20.9]	14.5 [10.3-18.8]	0.3 [0.2-0.4]	713 [425-1146]
	P. falciparum	23	4.5 [3.0-7.2]	16.6 [13.2-22.5]	0.2 [0.1-0.4]	237 [147-451]***
	P. vivax	26	4.8 [3.0-7.7]	17.2 [12.7-21.1]	0.2 [0.2-0.3]	393 [203-679]*
	P. malariae	9	4.9 [3.6-6.5]	18.5 [16.2-22.1]	0.2 [0.1-0.3]	348 [199-674]
	Mixed	7	3.1 [2.5-9.7]	17.9 [14.1-18.7]	0.3 [0.1-0.5]	287 [138-345]*
	P-value [e]		<0.0001	0.561	0.663	0.009
Sabah	Controls	27	21.4 [11.2-27.6]	18.2 [11.8-35.2]	1.1 [0.8-1.4]	316 [216-448]
	P. falciparum	12	6.0 [2.8-11.3]**	14.6 [7.5-34.5]	0.4 [0.1-0.6]****	391 [203-998]
	P. vivax	78	6.8 [4.4-13.9]****	14.0 [6.1-26.2]	0.8 [0.5-1.4]	124 [66-209]****
	P. knowlesi	92	10.5 [5.4-18.3]*	7.3 [4.0-13.2]****	0.6 [0.3-1.0]**	118 [70-283]****
	P-value [e]		<0.0001	<0.0001	<0.0001	0.004

Footnotes:

All values are median [interquartile range] unless otherwise indicated.

[a] Formula = ([%platelet-uRBC from flow data] ÷ 10^2 x [RBC count from analyzer] x 10^6) + ([%platelet-iRBC from flow data] ÷ 10^2 x [parasites per μL])

[b] Formula = (platelet count from analyzer x 10^3) ÷ (platelet-RBC per μL[a])

[c] Formula = ([%platelet-WBC from flow data] ÷ 10^2) x (WBC count from analyzer) x 10^3

[d] Formula = (platelet count from analyzer x 10^3) ÷ (platelet-WBC per μL[c])

[e] Kruskal-Wallis with Dunn's multiple comparisons test, significantly different to controls (****p<0.0001, ***p<0.0005, **p<0.005, *p<0.05).

Table 4. Platelet-associated parasite killing ($TUNEL^+PF4^+$ iRBC) between species and stages

Patient cohort	***Plasmodium* species**	***n* of samples analyzed**	**Median number parasites counted per sample [IQR]**	**Median % $TUNEL^+PF4^+$ iRBC [IQR]**	***P*-value [a]**	***P*-value [b]**
Papua	*P. falciparum*	50	54 [52-59]	5.6 [1.8-11.3]	<0.0001	-
	P. vivax	32	54 [51-58]	59.3 [45.4-81.1]	comparator	-
	P. malariae	11	45 [39-50]	14.3 [13.6-18.2]	0.009	-
	Mixed infection	6	59 [42-66]	14.9 [7.8-51.5]	0.150	-
	P. vivax (ring)	15	48 [28-61]	6.4 [1.1-16.1]	-	comparator
	P. vivax (mature)	16	59 [44-88]	63.6 [52.9-74.1]	-	<0.0001
	P. malariae (ring)	6	12 [16-18]	0 [0-3.5]	-	comparator
	P. malariae (mature)	9	40 [63-80]	6.3 [4.4-8.8]	-	0.007
Sabah	*P. falciparum*	14	95 [76-131]	10.6 [5.4-14.6]	0.043	-
	P. vivax	13	49 [21-104]	16.7 [13.5-39.1]	comparator	-
	P. knowlesi	15	95 [78-101]	14.2 [19.8-26.9]	0.717	-
	P. knowlesi (ring)	8	96 [92-103]	10.8 [8.0-24.3]	-	comparator
	P. knowlesi (mature)	5	78 [72-98]	14.9 [8.6-51.5]	-	0.354

Footnotes:

[a] *P*-values from Kruskal-Wallis with Dunn's multiple comparisons test for difference to *P. vivax*.

[b] *P*-values from Mann-Whitney test between intra-species ring and mature stages.

Abbreviations: IQR, interquartile range; PF4, platelet factor-4.

Figure Legends

Fig. 1. Platelet binding in clinical malaria blood samples. A) Photos of platelet-bound iRBC from patient Giemsa smears (black arrowhead = platelet). Images were taken at 1000x magnification using a Samsung Note-3 camera attached to an Olympus CX31 microscope. Scale bar = 5 μm. B) Representative flow cytometry gating strategy to measure platelet binding in a *P. falciparum* patient and healthy control. C) Frequency of platelet-bound uRBC by flow cytometry in malaria patients compared to controls in samples from Ci) Papua (controls *n*=17, *Pf n*=23, *Pv n*=26, *Pm n*=9, mixed *n*=7) and Cii) Sabah (controls *n*=28, *Pf n*=14, *Pv n*=85, *Pk n*=106) (Kruskal-Wallis, *significantly different to all other groups). D) Frequency of platelet-bound iRBC and uRBC by flow cytometry in samples from Di) Papua (*n* as per Ci) and Dii) Sabah (*n* as per Cii) (Wilcoxon test). E) Inverse correlation of platelet-bound iRBC with parasitemia in samples from Ei) Papua and Eii) Sabah (Spearman). Scatterplots indicate median ± interquartile range for each group. Parasitemia values are log transformed. Data presented in **Table S1**. Abbreviations: *Pf*, *P. falciparum*; *Pv*, *P. vivax*; *Pm*, *P. malariae*; *Pk*, *P. knowlesi*.

Fig. 2. PF4-associated parasite killing in clinical malaria samples. A) Representative immunofluorescent images from *Pf*, *Pv*, *Pk* and *Pm* patient blood smears illustrating PF4-associated parasite killing ($PF4^+TUNEL^+$ iRBC). Scale bars = 5 μm. Arrows and arrowheads indicate platelets and parasites, respectively. Images were taken at 630x magnification on an Axio Scope A1 fluorescent microscope coupled to an Axiocam ICm-1 CCD camera, or an Axio Observer inverted fluorescence microscope coupled to an Axiocam 503 monochrome camera. ZEN 2 software was used for image acquisition and processing (all from Carl Zeiss, Germany). B) Percentage of $PF4^+TUNEL^+$ parasites in clinical samples with *Pf* (Papua *n*=50, Sabah *n*=14),

Pv (Papua *n*=32, Sabah *n*=13), *Pm* (*n*=11), *Pk* (*n*=15) and mixed species infection (*n*=6). C) Comparison of intraerythrocytic PF4 ($PF4^+$) parasites as a percentage of dying ($TUNEL^+$) parasites in *Pf*, *Pv*, *Pm, Pk* and mixed species infection from Papua and Sabah (*n* as per panel B). D) Inverse correlation of $PF4^+TUNEL^+$ iRBC with parasitemia in *Pf* and *Pv* clinical samples (Spearman). E) Proportions of $PF4^+TUNEL^+$ rings versus mature stages in *Pv* (rings *n*=15, mature *n*=16), *Pm* (rings *n*=6, mature *n*=9) and *Pk* (rings *n*=8, mature *n*=5). F) Proportions of rings that were $PF4^+TUNEL^+$ and G) $PF4^+$ in *Pf* (Papua *n*=50, Sabah *n*=14), *Pv* (*n*=15), *Pm* (*n*=6) and *Pk* clinical samples (*n*=8). Scatterplots indicate median ± interquartile range for each group. Parasitemia values are log transformed. Kruskal-Wallis or Mann-Whitney test used for statistical comparisons. Data presented in **Table 4**. Abbreviations: *Pf*, *P. falciparum*; *Pv*, *P. vivax*; *Pm*, *P. malariae*; *Pk, P. knowlesi*.

Fig. 3. *In vitro* cultures of *P. knowlesi* are sensitive to platelets and PF4. The growth of A) *P. knowlesi* (*n*=4) *and* B) *P. falciparum* (*n*=3) treated with different platelet concentrations or Tyrodes buffer for 48 h. C) The growth of *P. knowlesi* treated with platelet lysate, with and without anti-PF4 antibodies or IgG isotype control (*n*=2). D) *P. knowlesi* PF4 dose response curve (*n*=2). E) The growth of *P. knowlesi* treated with platelets (60 million/mL), platelet lysate or PF4 (0.5 μM), and co-cultured in standard wells or Transwells (*n*=2). F) Micrographs showing platelets bound to uninfected and *P. knowlesi*-infected cells. G) Percent platelet binding to uninfected, *P. knowlesi* (*n*=4) or *P. falciparum* iRBC (*n*=3), determined by flow cytometry. H) Percent TUNEL-labelled ($TUNEL^+$) *P. knowlesi* parasites co-stained for PF4 ($PF4^+$) or not PF4-stained ($PF4^-$) (*n*=3). I) Micrographs showing a $PF4^+TUNEL^+$ *P. knowlesi* infected cell after platelet treatment. Scale bars = 5 μm. Images were taken at 630x magnification on an Axio

Observer inverted fluorescence microscope coupled to an Axiocam 503 monochrome camera with ZEN 2 software (Carl Zeiss, Germany). Bars indicate means of replicate data points. Kruskal-Wallis test or one-way ANOVA used for statistical comparisons: *$p<0.05$ and **$p<0.01$. Abbreviation: DIC, differential interference contrast.

Figure 1

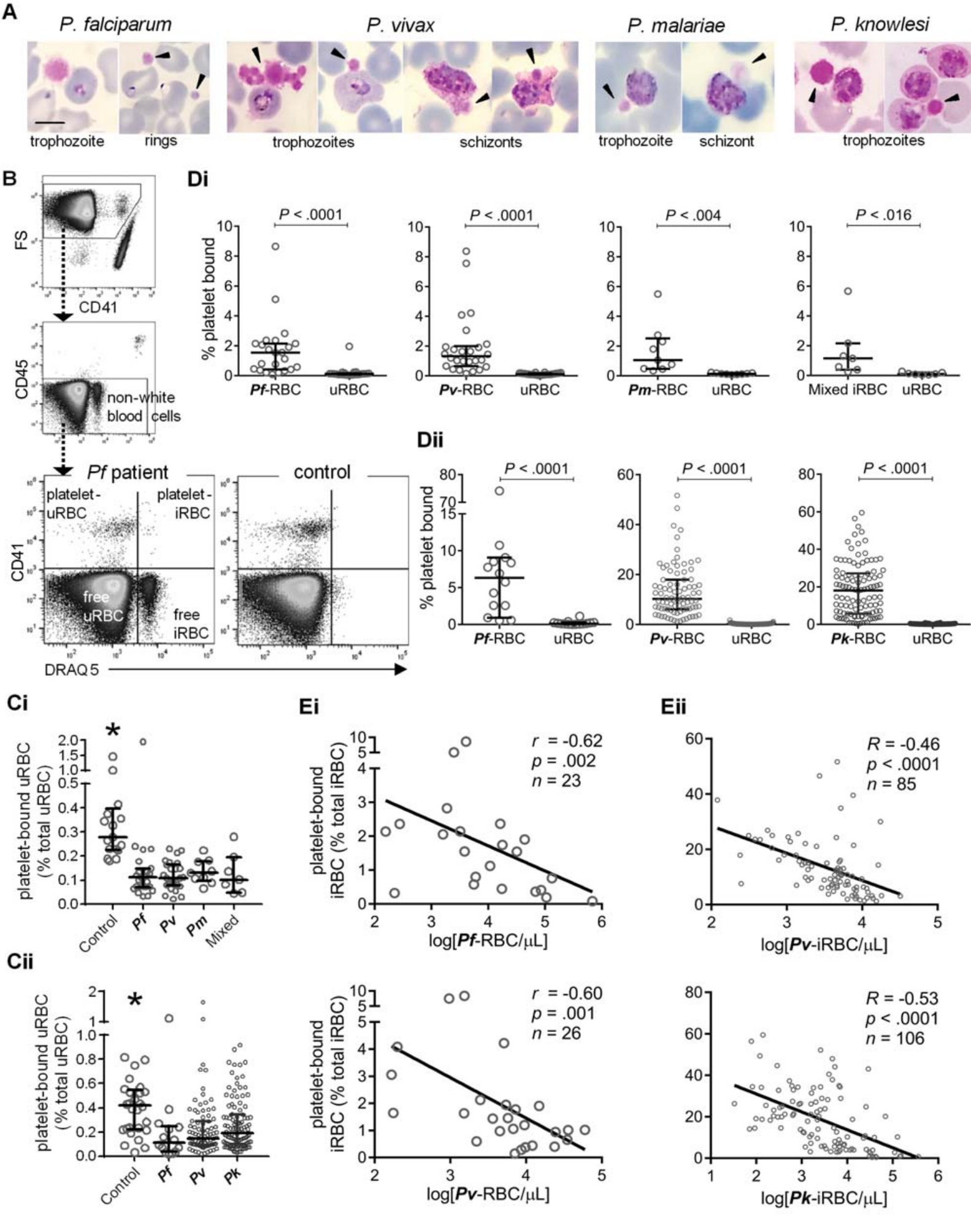

A
P. falciparum
P. vivax
P. malariae
P. knowlesi
trophozoite
rings
trophozoites
schizonts
trophozoite
schizont
trophozoites
B
FS
CD41
CD45
non-white blood cells
Pf patient
control
platelet-uRBC
platelet-iRBC
free uRBC
free iRBC
CD41
DRAQ5
Di
% platelet bound
P < .0001
P < .0001
P < .004
P < .016
Pf-RBC
Pv-RBC
Pm-RBC
Mixed iRBC
uRBC
Dii
% platelet bound
P < .0001
P < .0001
P < .0001
Pf-RBC
Pv-RBC
Pk-RBC
uRBC
Ci
platelet-bound uRBC (% total uRBC)
Control
Pf
Pv
Pm
Mixed
Cii
platelet-bound uRBC (% total uRBC)
Control
Pf
Pv
Pk
Ei
platelet-bound iRBC (% total iRBC)
r = -0.62
p = .002
n = 23
log[Pf-RBC/μL]
Eii
platelet-bound iRBC (% total iRBC)
R = -0.46
p < .0001
n = 85
log[Pv-iRBC/μL]
platelet-bound iRBC (% total iRBC)
r = -0.60
p = .001
n = 26
log[Pv-RBC/μL]
platelet-bound iRBC (% total iRBC)
R = -0.53
p < .0001
n = 106
log[Pk-iRBC/μL]

Figure 2

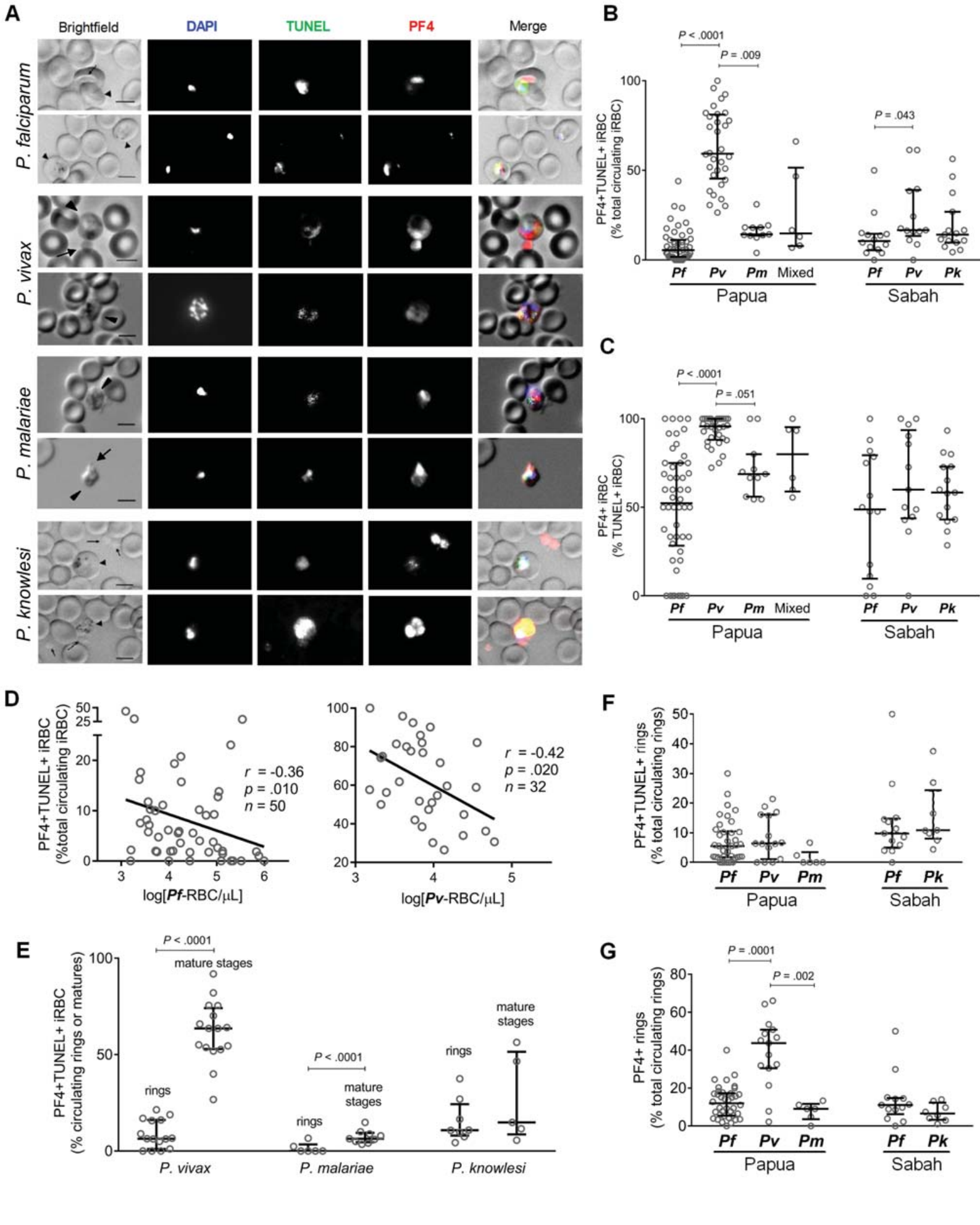
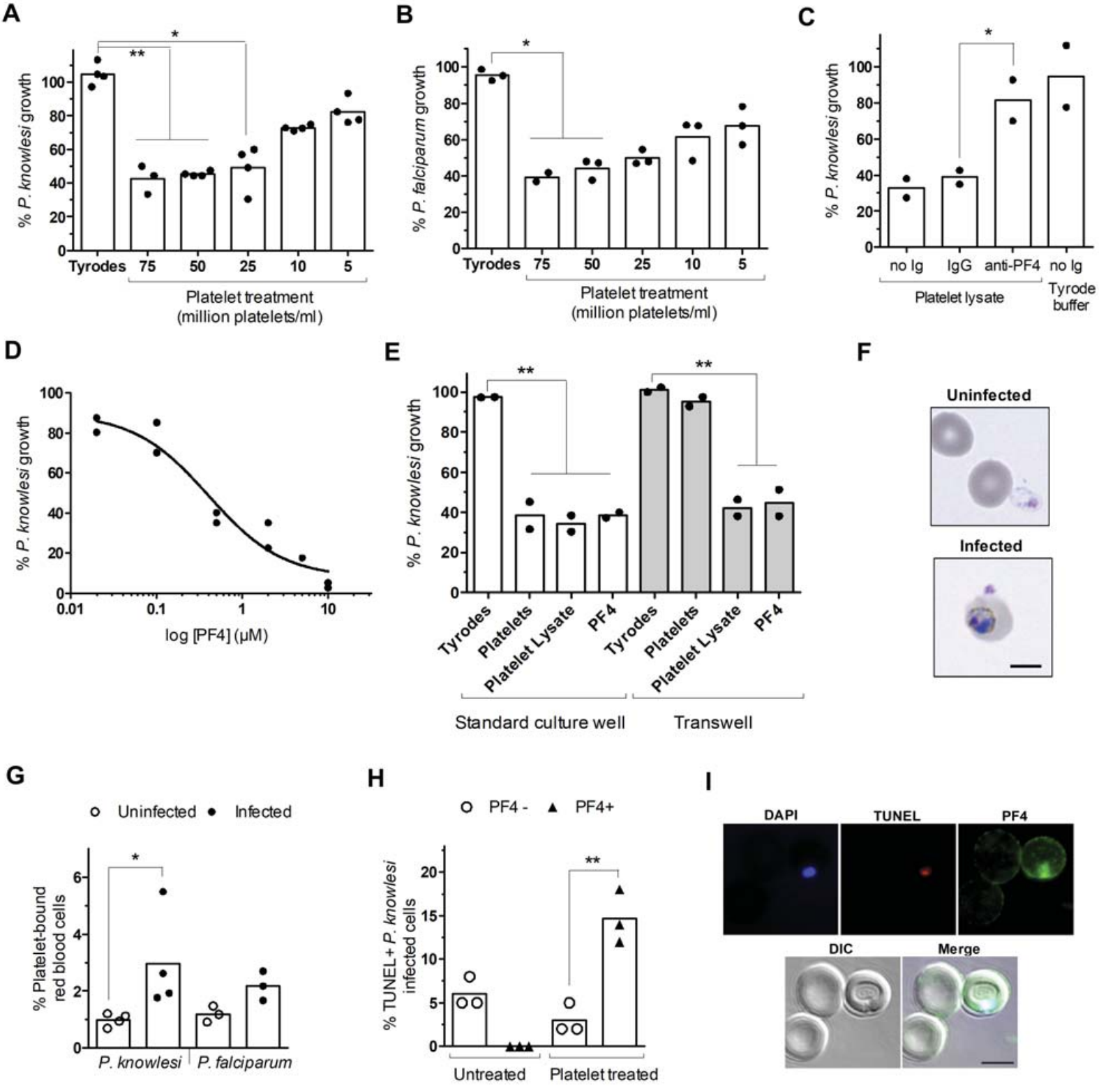

A
Brightfield
DAPI
TUNEL
PF4
Merge
P. falciparum
P. vivax
P. malariae
P. knowlesi
B
P < .0001
P = .009
P = .043
PF4+TUNEL+ iRBC (% total circulating iRBC)
Pf Pv Pm Mixed
Papua
Pf Pv Pk
Sabah
C
P < .0001
P = .051
PF4+ iRBC (% TUNEL+ iRBC)
Pf Pv Pm Mixed
Papua
Pf Pv Pk
Sabah
D
PF4+TUNEL+ iRBC (%total circulating iRBC)
r = -0.36
p = .010
n = 50
log[Pf-RBC/μL]
r = -0.42
p = .020
n = 32
log[Pv-RBC/μL]
E
P < .0001
mature stages
rings
P < .0001
mature stages
rings
rings
mature stages
PF4+TUNEL+ iRBC (% circulating rings or matures)
P. vivax
P. malariae
P. knowlesi
F
PF4+TUNEL+ rings (% total circulating rings)
Pf Pv Pm
Papua
Pf Pk
Sabah
G
P = .0001
P = .002
PF4+ rings (% total circulating rings)
Pf Pv Pm
Papua
Pf Pk
Sabah

Figure 3


A
% P. knowlesi growth
Tyrodes
75
50
25
10
5
Platelet treatment
(million platelets/ml)
B
% P. falciparum growth
Tyrodes
75
50
25
10
5
Platelet treatment
(million platelets/ml)
C
% P. knowlesi growth
no Ig
IgG
anti-PF4
no Ig
Platelet lysate
Tyrode buffer
D
% P. knowlesi growth
log [PF4] (μM)
E
% P. knowlesi growth
Tyrodes
Platelets
Platelet Lysate
PF4
Tyrodes
Platelets
Platelet Lysate
PF4
Standard culture well
Transwell
F
Uninfected
Infected
G
Uninfected
Infected
% Platelet-bound red blood cells
P. knowlesi
P. falciparum
H
PF4 -
PF4+
% TUNEL+ P. knowlesi infected cells
Untreated
Platelet treated
I
DAPI
TUNEL
PF4
DIC
Merge